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Fluorescence *In Situ* Hybridization (FISH) and Peptide Nucleic Acid Probe-Based FISH for Diagnosis of Q Fever Endocarditis and Vascular Infections

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ABSTRACT Endocarditis and vascular infections are common manifestations of persistent localized infection due to *Coxiella burnetii*, and recently, fluorescence *in situ* hybridization (FISH) was proposed as an alternative tool for their diagnosis. In this study, we evaluated the efficiency of FISH in a series of valve and vascular samples infected by *C. burnetii*. We tested 23 *C. burnetii*-positive valves and thrombus samples obtained from patients with Q fever endocarditis. Seven aneurysms and thrombus specimens were retrieved from patients with Q fever vascular infections. Samples were analyzed by culture, immunochemistry, and FISH with oligonucleotide and PNA probes targeting *C. burnetii*-specific 16S rRNA sequences. The immunohistochemical analysis was positive for five (17%) samples with significantly more copies of *C. burnetii* DNA than the negative ones ($P = 0.02$). FISH was positive for 13 (43%) samples and presented 43% and 40% sensitivity compared to that for quantitative PCR (qPCR) and culture, respectively. PNA FISH detected *C. burnetii* in 18 (60%) samples and presented 60% and 55% sensitivity compared to that for qPCR and culture, respectively. Immunohistochemistry had 38% and 28% sensitivity compared to that for FISH and PNA FISH, respectively. Samples found positive by both immunohistochemistry and PNA FISH contained significantly more copies of *C. burnetii* DNA than the negative ones ($P = 0.03$). Finally, PNA FISH was more sensitive than FISH (60% versus 43%, respectively) for the detection of *C. burnetii*. We provide evidence that PNA FISH and FISH are important assays for the diagnosis of *C. burnetii* endocarditis and vascular infections.

KEYWORDS infective endocarditis, vascular infections, *Coxiella burnetii*, Q fever, oligonucleotide probe, PNA probe, FISH

Endocarditis and vascular infection are the most common manifestations of persistent localized infection due to *Coxiella burnetii* (1). The incidence of endocarditis after acute Q fever in patients with valvulopathy has been estimated to be 39% (2). Q fever endocarditis is associated with surgery for 15% to 73% of patients, causes death in 5% to 65% of patients, and induces a large number of relapses when it is inadequately treated (3). The prognosis of *C. burnetii* endocarditis has considerably improved because of earlier diagnosis and appropriate dual-antibiotic therapy (4). Vascular infection is the second most frequent persistent presentation of Q fever, affecting a preexisting lesion, aneurysm, or vascular prosthesis (5). An increasing number of reports of *C. burnetii* vascular infections have been published in the last decade, mainly due to the Q fever epidemic in the Netherlands (6–8).

Q fever endocarditis and vascular infections are difficult to diagnose, primarily because *C. burnetii* is a fastidious bacterium. To date, its diagnosis mainly relies on

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serological examination by an indirect immunofluorescence assay (9). The analysis of resected valves by culture, quantitative PCR (qPCR), and immunohistochemistry is mostly performed in referent laboratories and shows variable performances (from 0% to 87% positivity), depending on the time when the resections are performed in relation to treatment (4). Similarly, in a series studying vascular complications of Q fever infections, culture and qPCR were used on vascular samples and presented 58% and 91% sensitivity, respectively (8). In addition, immunohistochemistry often fails to confirm the diagnosis, particularly as a result of early antibiotic treatment. In recent years, fluorescence *in situ* hybridization (FISH) has been proposed for the diagnosis of Q fever endocarditis using the pan-bacterial probe in both humans (10) and animals (11). Recently, the detection of *C. burnetii* in heart valve tissues by FISH has also been reported (12). FISH using specific probes targeting the 16S rRNA was previously used for the diagnosis of infective endocarditis due to other intracellular bacteria, including *Bartonella quintana* and *Tropheryma whipplei* (13). In addition, FISH using peptide nucleic acid probes (PNAs), synthetic homologs of nucleic acids, has been thought to be more sensitive than FISH with oligonucleotide probes and provides a rapid diagnosis of infectious diseases (14, 15). The objective of our study was to evaluate the efficiency of FISH for the diagnosis of Q fever endocarditis and vascular infections. To achieve this, we analyzed a series of valve and vascular samples infected with *C. burnetii* by using oligonucleotide and PNA probes, and we compared these results to those of molecular assays, culture, and immunohistochemical analysis.

MATERIALS AND METHODS

Samples. We tested a series of valves and thrombus samples collected from patients with endocarditis. We also analyzed a series of aneurysms and thrombus specimens from patients with vascular infections. All these samples were received from both hospitalized patients and outpatients throughout France in our Q fever reference center in Marseille between 2014 and 2017. Samples were frozen on dry ice (−80°C) and sent to our laboratory under sterile conditions, except from two cardiac valves that were received as formalin-fixed paraffin-embedded tissue sections.

DNA extraction and real-time PCR assay. The total genomic DNA was extracted using a DNA tissues kit on an EZ1 Advanced XL device (Qiagen, Courtaboeuf, France), as previously described (16). The qPCR assays were performed on a CFX96 device (Bio-Rad Clinical Diagnostics, Marnes-la-Coquette, France) using the Takyon No ROX probe 2× master mix UNG (Eurogentec, Anger, France), according to the manufacturer's instructions. The amplification of a housekeeping gene encoding beta-actin was used to assess the quality of DNA extraction, as previously described (17). *Coxiella burnetii* was detected by two amplification systems targeting the repeated sequences IS*1111* and IS*30A* (18). Sensitivity determination was performed, with a standard calibration curve using serial 10-fold dilutions (from 10^9 to 1 copy/5 μl) of a *C. burnetii* plasmidic control. Each dilution was tested using IS*1111* qPCR to express the threshold cycle (C_T) as the number of copies of IS*1111*/ml per sample (see Fig. S1 in the supplemental material).

Culture. All cardiac valves and vascular tissues, except the two samples mentioned above, were inoculated on human embryonic lung (HEL) fibroblasts following the shell vial assay, as previously described (19). The detection of *C. burnetii* growth was performed 20 and 30 days after inoculation by Gimenez staining, immunofluorescence, and qPCR targeting the IS*1111* and IS*30A* sequences.

Histologic analysis. Immunohistochemical analysis was performed on paraffin-embedded tissue sections with the anti-*C. burnetii* Cb100B10 mouse monoclonal antibody (20). The immunohistochemical procedure used the Ventana Benchmark autostainer (Ventana Medical Systems, Inc., Tucson, AZ). A negative control was performed using an irrelevant monoclonal antibody.

Fluorescence *in situ* hybridization. (i) FISH and PNA FISH procedures. "FISH" refers to all assays performed with oligonucleotide probes, and "PNA FISH" refers to all assays performed with PNA probes. Each sample was analyzed in duplicates with the two FISH assays in order to compare the efficiency of oligonucleotide probes versus that of PNA probes. Samples were also analyzed in the absence of any probe to check for autofluorescence. For each sample, FISH was performed on 3-μm-thick formalin-fixed paraffin-embedded tissue sections. The paraffin was removed from slices by incubating for 10 min at 65°C and then for 10 min in a substitute xylene solution. The tissue was rehydrated in a descending ethanol series (100%, 70%, and 50%; 5 min each), rinsed in water, and air dried. The FISH procedure was performed as previously described (21). For PNA FISH, the slides were incubated at 55°C for 90 min with hybridization buffer containing 10% (wt/vol) dextran sulfate, 0.1% (vol/vol) Triton X-100, 50 mM Tris-HCl (pH 8.0), 30% formamide, and 500 nM PNA probes. The hybridization step can be performed using a hybridizer device (Dako; Agilent, Santa Clara, CA) for both FISH assays. Regarding the PNA FISH washing step, the slides were incubated at 55°C for 30 min in washing buffer containing 5 mM Tris base (pH 10), 15 mM NaCl, and 1% (vol/vol) Triton X-100. The slides were rinsed once again with water and then dried and mounted with mounting medium containing an antifade agent and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) to visualize microorganisms and host cell nuclei. The slides were visualized under confocal laser scanning microscopes (CLSMs) by using the Leica SP5 resonant scanner with appropriate filter sets for fluorescence and a 60× oil immersion objective, as previously described (22). L929 cells and

TABLE 1 Comparison of microbiological assays for the detection of *C. burnetii* in cases of endocarditis and vascular infections

Sample	No. of samples	No. (%) of positive samples			
		Culture	IHC	FISH	PNA FISH
Endocarditis					
Aortic valves	10	4 (40)	3 (30)	6 (60)	9 (90)
Mitral valves	6	5 (83)	1 (17)	4 (67)	5 (83)
Tricuspid valve	1	1 (100)	1 (100)	1 (100)	1 (100)
Hancock bioprosthesis	1	0	0	0	0
Prosthetic heart valves	4	3 (75)	0	1 (25)	2 (50)
Thrombus	1	1 (100)	0	0	0
Vascular infection					
Aneurysms	6	5 (83)	0	1 (17)	1 (17)
Thrombus	1	1 (100)	0	0	0
Total	30	20 (71[a])	5 (17)	13 (43)	18 (60)

[a]The percentage was calculated from 28 samples, as two cardiac valves were received as formalin-fixed paraffin-embedded tissue sections and culture was not performed.

mouse tissues infected with *C. burnetii* were used as positive controls. Noninfected L929 cells and noninfected human tissues were used as negative controls for FISH.

(ii) Oligonucleotide probes. We used the EUB338 probe (23) as a positive control and the probe NonEUB (24), which is the antisense probe of EUB338, as a negative control for nonspecific binding. A specific probe for *C. burnetii* targeting the 16S rRNA (CB-189; 5′-CCGAAGATCCCCCGCTTTGC-3′) was designed, and to assess its specificity, we compared specific CB-189 probe sequences with those of all 16S rRNA entries in the probeBase and GenBank databases showing 100% homology only with the *C. burnetii* 16S sequence. The three sequences (EUB388, NonEUB, and CB-189) were synthetized as oligonucleotide probes and PNA probes by Eurogentec. EUB338 probes (oligonucleotide and PNA) were labeled with Alexa Fluor 555, NonEUB probes were labeled with Alexa Fluor 647, and CB-189 probes were labeled with Alexa Fluor 488.

Statistical analysis. For data comparison, a χ^2 test was performed using EpiInfo version 6.0 software (Centers for Disease Control and Prevention, Atlanta, GA, USA). A P value of <0.05 was considered significant.

RESULTS

Among patients diagnosed with Q fever endocarditis, we analyzed 22 (96%) valve samples and one (4%) thrombus. We also tested six (86%) aneurysms and one (14%) thrombus obtained from patients with Q fever vascular infection (Table 1). All samples were positive for *C. burnetii* by the two qPCR systems targeting the IS*1111* and IS*30A* repeated sequences, respectively.

We isolated *C. burnetii* from 14 (67%) samples from patients with endocarditis and from 6 (86%) samples from patients with vascular infection (Table 1). Culture-positive samples presented significantly more log10 *C. burnetii* DNA copies (7.4 $\pm$ 1.5) than the culture-negative samples (6.7 $\pm$ 1) ($P = 0.02$). Compared to qPCR, culture showed a sensitivity of 67% for the diagnosis of *C. burnetii* endocarditis and 86% for the diagnosis of *C. burnetii* vascular infections.

Histologic analysis. Immunohistochemical analysis was positive for 5 (22%) samples obtained from patients with endocarditis, and all samples from patients with vascular infections were negative (Table 1). None of the controls showed immunoreactivity, confirming the specificity of the antibody used. In Q fever endocarditis, bacteria were visualized as coarse granular immunopositive material in the macrophage cytoplasm (Fig. 1). *Coxiella burnetii* was usually visualized within regions of inflammation as small focal collections of infected mononuclear cells. An immunohistochemical analysis of positive samples presented significantly more log10 *C. burnetii* DNA copies (9.1 $\pm$ 1) than samples tested negative by immunohistochemical analysis (7 $\pm$ 1.5) ($P = 0.02$). Immunohistochemistry was less sensitive than qPCR (17%) and culture (25%) for the diagnosis of Q fever endocarditis. Regarding the diagnosis of vascular infections, the sensitivity was 0% compared to that of qPCR or culture.

FISH. *Coxiella burnetii* was detected by FISH in 12 (52%) valve specimens from patients with endocarditis (Table 1; Fig. 2). Only one (14%) aneurysm was found positive

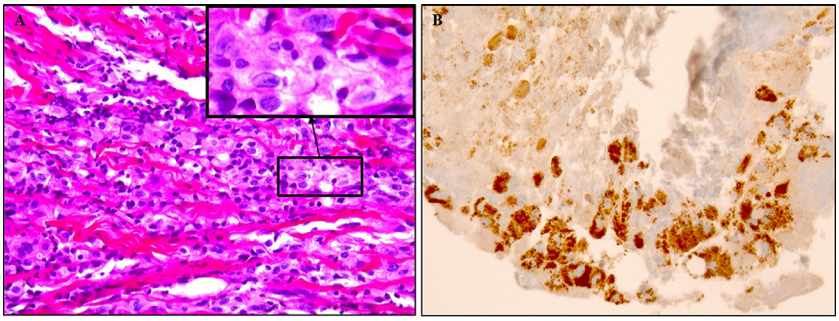

FIG 1 Histological analysis of a cardiac valve from a patient with Q fever endocarditis. (A) The image shows the abundant inflammatory infiltrate in valvular tissue, composed mainly of macrophages (hematoxylin-eosin-saffron; ×200 magnification). (B) Immunohistochemical detection of *C. burnetii* in a cardiac resected valve from a patient with Q fever endocarditis using a monoclonal antibody and hematoxylin counterstain. Note the intracellular location of the bacteria in the macrophage cytoplasm (×200 magnification).

by FISH (Fig. 3A). FISH-positive samples did not present more log10 *C. burnetii* DNA copies (7.6 ± 2) than the negative ones (7 ± 1) ($P = 0.1$) (Fig. 4A). In total, eight specimens were positive by culture and FISH; three were FISH positive but culture negative (Table 1). There was no difference for log10 *C. burnetii* DNA copies between samples that were positive by both immunohistochemistry and FISH (9.1 ± 1) and samples found positive by FISH but negative by immunohistochemistry (7 ± 2.2) ($P =$

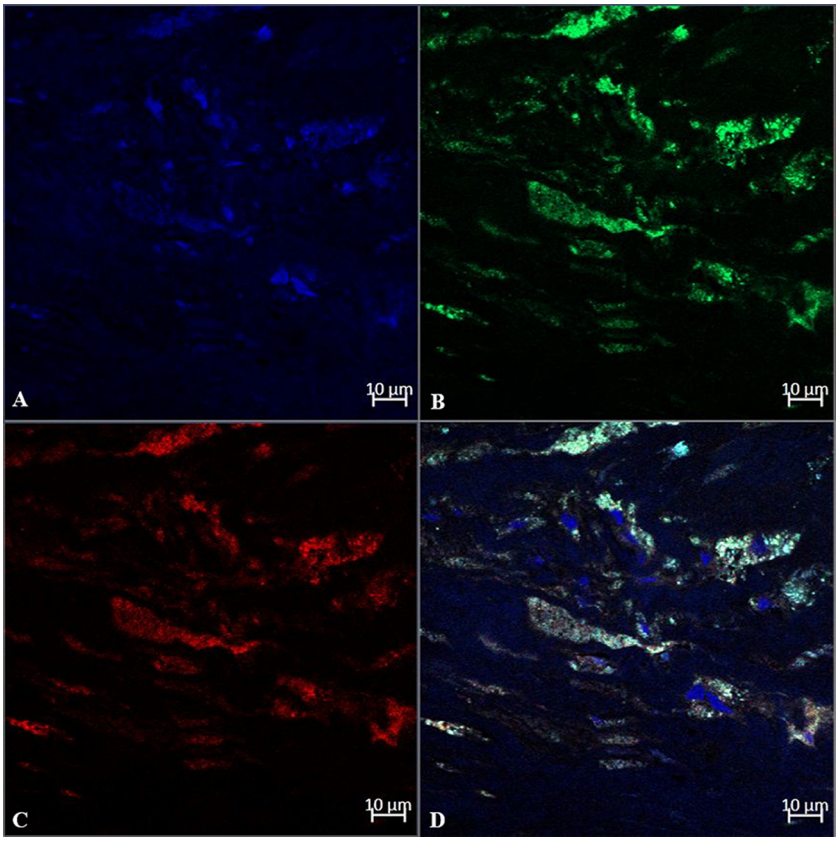

FIG 2 Detection of *C. burnetii* in a mitral valve by FISH. The images correspond to the nuclei counterstained with DAPI in blue (A), the green channel (Alexa 488) showing hybridization of oligonucleotide probes CB-189 (B), the red channel (Alexa 550) showing hybridization of the universal probe EUB338 (C), and the merged signals (D). Bacteria are visualized directly in infected cells, mainly in the intracytoplasmic area, and appear in clusters as multiple rounded structures.

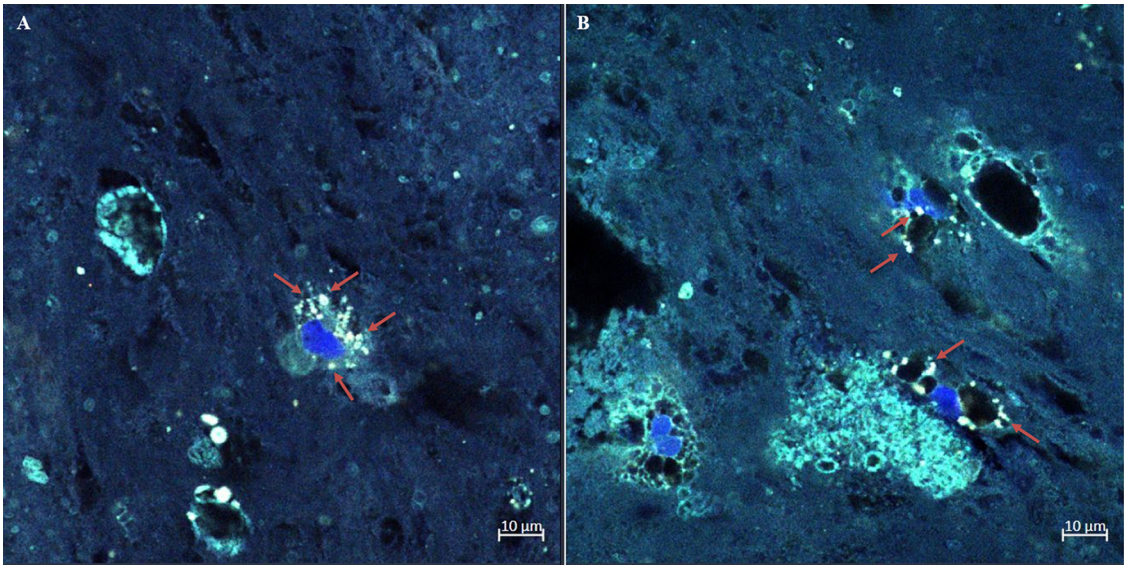

FIG 3 Detection of *C. burnetii* in aneurysm by FISH (A) and PNA FISH (B).

0.1). FISH showed a sensitivity of 43% and 40% to that of qPCR and culture, respectively. Finally, immunohistochemistry had 38% sensitivity compared to that of FISH.

PNA FISH. *Coxiella burnetii* was detected by PNA FISH assay in 17 valves samples (Table 1; Fig. 5). As for vascular infections, PNA probes detected *C. burnetii* in one (14%) aneurysm sample (Table 1; Fig. 3B). PNA FISH-positive samples did not present significantly more log10 *C. burnetii* DNA copies (7 ± 1.7) than the negative ones (7 ± 1.2) ($P = 0.6$) (Fig. 4B). In total, 11 samples were found positive by culture and PNA FISH; five were PNA FISH positive but culture negative. We found significantly more log10 *C. burnetii* DNA copies on samples that were positive by both immunohistochemistry and PNA FISH (9.1 ± 1) than on samples positive by PNA FISH and immunohistochemistry negative (7 ± 1.8) ($P = 0.03$). PNA FISH assays presented a sensitivity of 60% and 55% compared to that of qPCR and culture, respectively. Moreover, PNA FISH was more efficient than FISH (60% versus 43%, respectively) for the detection of *C. burnetii*. Finally, immunohistochemistry had 28% sensitivity compared to that of PNA FISH.

DISCUSSION

We evaluated the efficiency of FISH for the diagnosis of *C. burnetii* endocarditis and vascular infections in comparison to molecular, culture, and histologic analyses. To our

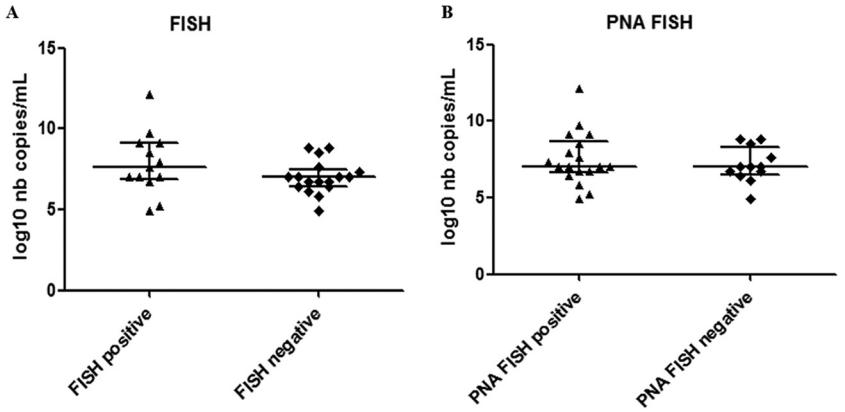

FIG 4 Comparison of IS*1111* log10 DNA copies in cases that were FISH positive and FISH negative (A) and PNA FISH positive and PNA FISH negative (B).

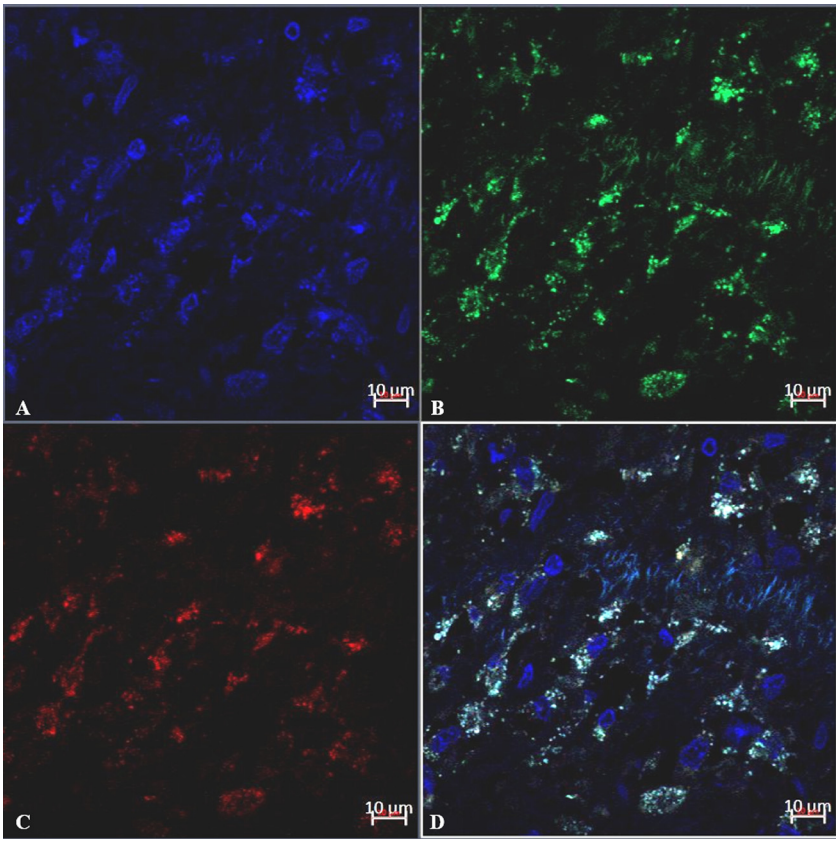

FIG 5 Detection of *C. burnetii* in a mitral valve by PNA FISH. The images correspond to the nuclei counterstained with DAPI in blue (A), the green channel (Alexa 488) showing hybridization of specific *C. burnetii* probe CB189 (B), the red channel (Alexa 550) showing hybridization of the universal probe EUB338 (C), and the merged signals (D). Bacteria are visualized directly in infected cells, mainly in the intracytoplasmic area, and appear in clusters as multiple rounded structures.

knowledge, this study is the first to evaluate the efficacy of FISH and PNA FISH for the diagnosis of Q fever endocarditis and vascular infections in a series of samples. Previously published studies have already shown that the autofluorescent particles present in tissues could produce false-positive results (12, 25, 26). To overcome this problem, we routinely included many negative and positive controls in each assay, processed in the same way as the test samples. In addition, all specimens were analyzed in duplicates by two experienced individuals. A possible limitation of this study was that histological analyses were performed on a 3-μm tissue sections, which contained a small tissue cell quantity or small inflammatory cell quantities. *Coxiella burnetii* infections can be localized in a small area of tissue (27, 28), and it is possible that the analysis of more different biopsy sections could potentially increase the sensitivity of both FISH and immunohistochemical analyses.

To date, immunohistochemistry is commonly used for the diagnosis of localized *C. burnetii* infections, and in a previous study of patients suffering from *C. burnetii* endocarditis, this assay presented 27% sensitivity (29). However, we proved that both FISH and PNA FISH gave better results than immunohistochemistry for the diagnosis of *C. burnetii* endocarditis and vascular infections. Recently, FISH and molecular assays also enabled the diagnosis of Q fever endocarditis in a patient with severe destruction of the aortic valve with perivalvular abscess formation and cardiac failure (10). Similarly, Aistleitner et al. diagnosed a case of Q fever endocarditis by using FISH as well (12). Finally, FISH has been used for the diagnosis of *C. burnetii* endocarditis in a series of cattle samples (11). However, none of the cases examined for *C. burnetii* by FISH were found to be positive when using the pan-bacterial or *C. burnetii*-specific probes, although molecular assays were positive.

We found that PNA FISH was better for the diagnosis of *C. burnetii* endocarditis and vascular infections than FISH with oligonucleotide probes and immunohistochemistry. PNA FISH was developed to provide a rapid and accurate diagnosis of infectious diseases with more sensitivity and specificity than FISH (30). As PNA is not charged, electrostatic repulsion does not occur when samples are hybridized with a complementary sequence; thus, the PNA/RNA duplexes have a mating specificity and a much higher stability than natural DNA/RNA duplexes (31). PNA FISH was used for detection of intracellular and fastidious bacteria, such as *Salmonella* spp. in blood and feces samples, for the identification of *Mycobacterium avium* and *Mycobacterium tuberculosis* in clinical specimens or for the detection of *Helicobacter pylori* in gastric biopsy specimens (32–34). PNA FISH has proven to be a highly sensitive and specific assay to identify the most common extracellular Gram-negative bacilli, such as *Escherichia coli* or *Klebsiella pneumoniae*, and extracellular Gram-positive cocci, such as *Enterococcus faecalis* from bloodstream infection (35, 36). The limitation of PNA FISH is that, although faster and more sensitive than FISH, PNA probes are nevertheless more expensive, which may explain why most laboratories still use oligonucleotide probes. Finally, in this study, both PNA FISH and FISH demonstrated their efficacies by being much faster than culture and enabling the diagnosis of diseases in five culture-negative patients.

In conclusion, we provide evidence that both PNA FISH and FISH are important assays for the diagnosis of *C. burnetii* endocarditis and vascular infections. Our results show that these assays are better than the immunohistochemistry that is now used for the diagnosis of *C. burnetii* localized infections. Although this needs to be confirmed, we believe that these assays might be effective for the diagnosis of *C. burnetii* in other tissue samples as well. From now on, we will be the first referent laboratory that routinely applies PNA FISH to tissue samples for the diagnosis of *C. burnetii* infections. This technique will not replace the other already approved diagnostic techniques, but we believe that it can be used as an important complementary tool when results are divergent.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00542-18.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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The authors declare that they have no conflicts of interest.

This study was approved by the ethics committee of the Méditerranée Infection foundation under number 2016-025. No informed consent was required as it was a retrospective study.

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